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(54) Title: METHOD FOR GENERATING GENE EXPRESSION PROFILES

(57) Abstract

The present invention provides a new method for producing gene expression profiles from a selected set of cells. This method combines and utilizes known methods in a novel way to produce the gene expression profiles. These gene expression profiles are useful for the identification of differentially expressed genes in specific cells.

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TITLE OF THE INVENTION

METHOD FOR GENERATING GENE EXPRESSION PROFILES

BACKGROUND OF THE INVENTION

Gene expression profiles of thousands of genes can now be examined *en masse* via cDNA and oligonucleotide microarrays (reviews [1-3]). Recently, studies have been reported that examined gene expression changes in yeast [4] [5] as well as in mammalian cell lines [6], primary cells [7] and tissues [8].

However, present applications of microarray technology do not include the study of gene expression from individual cell types residing in a given tissue/organ (i.e., *in-situ*). Such studies would greatly facilitate our understanding of the complex interactions that exist in-vivo between neighboring cell types in normal and disease states. The present invention demonstrates that gene expression profiles from adjacent cell types can be successfully obtained by integrating the technologies of laser capture microdissection [9] (LCM) and T7-based RNA amplification [10] with cDNA microarrays [11].

SUMMARY OF THE INVENTION

The present invention provides a method for the reproducible measurement and assessment of the expression of specific messenger RNA's in a specific set of cells. This method combines and utilizes the known techniques of laser capture microdissection, T-7 based RNA amplification, production of cDNA from the amplified RNA, and DNA microarrays containing immobilized DNA molecules for a wide variety of specific genes to produce a profile of gene expression analysis for very small numbers of specific cells in a new way. The desired cells are individually identified and attached to a substrate by the laser capture technique, and the captured cells are separated from the remaining cells. RNA is then extracted from the captured cells and amplified about one million-fold using the T7-based amplification technique, and, optionally, cDNA is prepared from the amplified RNA. A wide variety of specific DNA molecules are prepared which hybridize

with specific nucleic acids of interest which may or may not be present, or are present at some level in the captured cells, and the DNA molecules are immobilized on a suitable substrate to form the microarray. The cDNA made from the captured cells is applied to the microarray under conditions that allow hybridization of the cDNA to the immobilized DNA on the array. The expression profile of the captured cells is obtained from the analysis of the hybridization results using the amplified RNA or, optionally, cDNA made from the amplified RNA of the captured cells, and the specific immobilized DNA molecules on the microarray. The hybridization results demonstrate, for example, which genes of those represented on the microarray as probes, are hybridized to cDNA from the captured cells, and/or the amount of specific gene expression. The hybridization results represent the gene expression profile of the captured cells. The gene expression profile of the captured cells can be used to compare with the gene expression profile of a different set of captured cells, and the similarities and differences provide useful information for determining the differences in gene expression between different cell types, and differences between the same cell type under different conditions.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 - Laser capture microdissection (LCM) from 10 μm Nissl-stained sections of adult rat large and small dorsal root ganglion (DRG) neurons is shown. The red arrows indicate DRG neurons to be captured (top panel). The middle and bottom panels show successful capture and film transfer respectively. Scale bar = 200 μm.

Figure 2, Panels A and B - cDNA microarray expression patterns of small (S) and large (L) neurons is shown. In 2A is an example of the cDNA micraoarray data obtained. Boxed in white is an identical region of the microarray for L1 and S1 samples that is enlarged (shown directly below). In 2B, scatter plots showing correlation between independent amplifications of S1 vs. S2, S1 vs. S3, L1 vs. L2 and L (L1 and L2) vs. S (S1, S2 and S3).

Figure 3, Panels A and B – Representative fields of radioisotopic *in situ* hybridization of rat DRG with selected cDNAs is shown. The sections were Nissl-counterstained. The left panel (panel A) shows results with radiolabled probes encoding neurofilament-high (NF-H), neurofilament-low (NF-L) and β-1 subunit of the voltage-gated sodium channel (SCNβ-1). Red arrows in the left panel denote identifiable small neurons. The right panel (panel B) shows representative fields from radiolabeled probes encoding calcitonin gene-related product (CGRP), voltage gated Na channel (NaN) and phospholipase C delta 4 (PLC). Red arrows in the right panel denote identifiable large neurons. Large red arrowhead denotes a large neuron which is also labeled. Scale bar = 100 μm.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a new method for the generation of gene expression profiles in a specific chosen set of cells. The method of the present invention utilizes known techniques that are combined in a novel way to provide a methodology for accurately isolating only the cells that are desired for the analysis. This is accomplished through laser capture dissection in which individual cells from a mixed population of cells, such as an organ or tissue sample, become attached to a substrate by laser energy applied to specific cells selected for capture. The captured cells are separated from the non-captured cells to provide a set of cells that were specifically selected for inclusion within the set. Selection criteria can be any characteristics of the cells that are desired for inclusion within the set. Using this technique a set of cells is collected that have the desired characteristics, and represent a uniform collection of cells. The captured cells are then used to produce extracted RNA. The RNA is extracted from the captured cells using known techniques. This extracted RNA is then amplified using known techniques to about one million-fold amplification, and the amplified RNA is, optionally, used to produce cDNA using known techniques.

To demonstrate this integration of technologies, the differential gene expression between large and small-sized neurons in the dorsal root ganglia (DRG) was examined. In general, large DRG neurons are myelinated, fast-conducting and transmit mechanosensory

information, while small neurons are unmyelinated, slow-conducting and transmit nociceptive information[12]. This system was chosen because: (1) numerous differentially expressed genes (small vs. large) have been previously reported; thus the success of this experiment could be assessed and (2) many small and large neurons are adjacent to each other, thus testing whether individual neurons can be cleanly captured.

As shown in Figure 1, large (diameter of >40μm) and small (diameter < 25μm) neurons were cleanly and individually captured via LCM from 10 μm sections of Nissl-stained rat DRGs. For this study, two sets of 1000 large neurons and 3 sets of 1000 small neurons were captured for cDNA microarray analysis.

RNA amplification is reproducible between individual sets of neurons

RNA was extracted from each set of neurons and linearly amplified an estimated 10^6-fold via T7 RNA polymerase. Once amplified, three fluorescently labeled probes were synthesized from an individually amplified RNA (aRNA) and hybridized in triplicate to a microarray (a.k.a. chip) containing 477 cDNAs (see chip design described below) plus 30 cDNAs encoding plant genes (for the determination of non-specific nucleic acid hybridization). Expression in each neuronal set (designated as S1, S2, and S3 for small and L1 and L2 for large neurons) was thus monitored in triplicate, requiring a total of 15 microarrays. The quality of the microarray data is exemplified in Figure 2A which shows pseudo-color arrays, one resulting from hybridization to probes derived from neuronal set S1 and the other from neuronal set L2. In Figure 2A, the enlarged part of the chip shows some differences in fluorescence intensity (i.e., expression levels) for particular cDNAs and demonstrates that spots containing the different cDNAs are relatively uniform in size and that background between spots is relatively low.

To determine whether a signal corresponding to a particular cDNA is reproducible between different chips, for each neuronal set, the coefficient of variation was calculated (CV or standard deviation/mean X 100%). From these values, the overall average CV for all 477 cDNAs per neuronal set was calculated to be: 15.81%=S1, 16.93% =S2, 17.75% = S3, 20.17 % = L1 and 19.55% = L2.

More importantly, independent amplifications (~10^6-fold) of different sets of the same neuronal subtype yielded quite similar expression patterns. For example, the correlation of signal intensities between S1 vs. S2 was $R^2 = 0.9688$, and between S1 vs. S3 was $R^2 = 0.9399$ (Figure 2B). Similar results were obtained between the two sets of large neurons: $R^2 = 0.929$ for L1 vs. L2 (Figure 2B).

Conversely, a comparison between all three small neuronal sets (S1, S2 and S3) versus the two large sets (L1 and L2) yielded a much lower correlation ($R^2 = 0.6789$), demonstrating as expected that a subset of genes are differentially expressed between the two neuronal subtypes (Figure 2B).

Differential gene expression is demonstrated between small and large neurons

To identify the mRNAs that are differentially expressed between large and small neurons, all 477 cDNAs were examined and those with 1.5-fold or greater differences (at $P<0.05$) were sequenced and are shown in Table 1 and 2. Amongst the collection of cDNAs on the microarray, it was found that more mRNAs preferentially expressed in small neurons (14 mRNAs in large versus 27 in small); this may simply reflect the set of cDNAs used on this chip.

To confirm the observed differential gene expression, *in-situ* hybridization was performed with a subset of these cDNAs.

For small neurons, five mRNAs were examined which encoded the following: fatty acid binding protein (GenBank accession # M13501), NaN (sodium voltage-gated channel, AF059030), phospholipase C delta-4 (U16655), CGRP (L00111) and annexin V (82462). All five mRNAs are preferentially expressed in small neurons (three of the five are shown in Figure 3, see Table 3 for all five). This was based on quantitative measurements in which was measured for a given mRNA the (1) overall intensity of signal in small and large neurons and (2) percentage of cells labeled within the total population of either small or large neurons (Table 3).

The results confirmed *in-situ* hybridization studies for NaN mRNA [13] and CGRP mRNA[14] and are consistent with immunofluorescent studies with annexin V[15].

Phospholipase C delta 4 has previously been shown to be induced in S-phase of the cell cycle and reside in the nucleus[16]. Given that neurons are post-mitotic, this observation suggests that this enzyme may play a different role in a subset of small neurons.

For large neurons, three cDNAs were examined, neurofilaments NF-L (M25638) and NF-H (J04517) as well as the beta-1 subunit (M91808) of voltage-gated sodium channels. As shown in Figure 3 and Table 3, preferential expression of these mRNAs was found in large DRG neurons. Recent *in-situ* hybridization studies have also demonstrated preferential expression in large neurons for these three mRNAs.[14 17] In addition, previous *in-situ* hybridization studies are in agreement with this cDNA chip data for the differential expression in small and large neurons of P2X3 receptor mRNA (X90651), NF-middle (J04517), hsp 27 [18]and peripherin (M26232).[14, 19, 20]. One report, however, finds no differences between small and large neurons for peripherin mRNA expression.[14]

In general, for a given mRNA, the cDNA chip data and the results from the *in-situ* hybridization studies are in agreement. However, in most cases, *in-situ* hybridization studies indicate much greater differences in expression between small and large than what is observed from cDNA chip data (Table 1 and 2 vs. Table 3). This is particularly apparent for low intensity signals. For example, phospholipase C delta 4 expression was found almost exclusively in small neurons (Table 3) although the chip data indicates only a 2.76-fold difference in expression (Table 2). In most part, this can be explained by the fact that background signal due to non-specific nucleic acid hybridization (i.e., hybridization signal from plant cDNAs) has not been subtracted from each cDNA intensity.. As indicated below, the 75-percentile value background signal for plant cDNAs is 48.68 for small and 40.94 for large neuronal sets. Thus the ratio of expresion of small to large neurons for phospholipase C delta 4 would go from a 2.76-fold difference to ~22-fold difference if the "non-specific" background is subtracted. The reason for not subtracting this background is that it can also lead to very large and potentially spurious fold-differences as the denominator (i.e., intensity of signal minus 75% plant value) approaches zero.

Overall, of the 41 mRNAs preferentially expressed in either large or small neurons, similar results have been demonstrated for 12 of these mRNAs via *in-situ* hybridization studies. This level of success suggests that most of the other 30 mRNAs are also differentially expressed in small or large neurons.

Building gene expression databases containing cell type specificity

It was demonstrated that by integrating LCM, aRNA and cDNA chips, one can successfully screen different cell types obtained from *in-situ* and subsequently identify differential gene expression. Although this study has identified mRNAs with differential expression within DRG neurons, there exists a great deal more heterogeneity within DRG neurons beyond simply small and large. For example, within small neurons (i.e., nociceptive neurons) there is heterogeneity with respect to gene expression, which presumably reflects, at least in part, the different sensory modalities transmitted. To approach this more complicated heterogeneity, the coupling of immunocytochemistry to LCM followed by aRNA and DNA chip analysis can be done. In addition, chips containing a larger number of cDNAs (i.e., >10,000) can be completed to more fully identify differential gene expression between large and small neurons.

The results shown herein demonstrate that expression profiles generated via this integration of known technologies can not only be useful for screening cDNAs, but also, more importantly, to produce databases that contain cell type specific gene expression. Cell type specificity within a database will give an investigator much greater leverage in understanding the contributions of individual cell types to a particular normal or disease state and thus allow for a much finer hypotheses to be subsequently generated. Furthermore, genes, which are coordinately expressed within a given cell type, can be identified as the database grows to contain numerous gene expression profiles from a variety of cell types (or neuronal subtypes). Coordinate gene expression may also suggest functional coupling between the encoded proteins and therefore aid in one's attempt to determine function for the vast majority of cDNAs currently cloned.

Table 1

mRNA enriched in large DRG neurons. [GB] gene bank accession number; [Mean] mean intensity of DNA chip microarrays; [S.E.M.] standard error of mean; [Ratio] mean intensity ratio of large DRG vs. small DRG neurons; [*] mean intensity not significantly different (p >0.05) from 75% of plant value.

Table 2

mRNA enriched in small DRG neurons. [GB] gene bank accession number; [Mean] mean intensity of DNA chip microarrays; [S.E.M.] standard error of mean; [Ratio] mean intensity ratio of small DRG vs. large DRG neurons; [*] mean intensity not significantly different (p >0.05) from 75% of plant value.

Table 3

In situ hybridization of selected cDNA clones. [Intensity] = estimated mRNA expression level per cell as follows: [-] no above background expression; [±] weak expression; [+] mild expression; [++] moderate expression; [+++] strong expression. [%] = percentage of DRG neurons expressing above background the mRNA of interest.

Table 1

Clone ID	GB	Description	Small DRG		Large DRG	
			Intensity	% of Labeled	Intensity	% of Labeled
192393	M25638	Rat smallest neurofilament protein (NF-L)	±	100%	+++	100%
192157	J04517	Rat high molecular weight neurofilament (NF-H)	± / -	21.40%	+++	98.60%
192424	M91808	Rattus norvegicus sodium channel beta-1	± / -	10%	++	96.30%
192273	M13501	Rat liver fatty acid binding protein,	+ / ++	62.20%	+ / -	1%
192294	AF059030	Rattus norvegicus voltage-gated Na channel NaN	++ / +	96.70%	+ / -	4.20%
192199	D42137	Rat annexin V gene	+ / ++	95.00%	+ / ++	74.00%
192207	U16655	Rattus norvegicus phospholipase C delta-4	++	42.20%	-	0%

191857	L00111	Rat CGRP	+++ / ++	83.70%	++ / -	9.40%

Table 2

192294	AF059030	Rattus norvegicus voltage-gated Na channel alpha subunit NaN	161.34±20.07	51.3±12.99*	3.15	0.0005
192195	D86642	Rat mRNA for FK506-binding protein	496.33±40.11	158.8±35.13	3.13	0.0005
192207	U16655	Rattus norvegicus phospholipase C delta-4	146.33±10.03	53.06±4.23	2.76	0.0005
192163	X90651	R. norvegicus P2X3 receptor	390.28±10.4	164.81±26.22	2.37	0.0005
191858	S69874	C-FABP=cutaneous fatty acid-binding protein [rat)	448.26±30.01	196.97±18.68	2.28	0.0005
192139	D45249	Rat proteasome activator rPA28 subunit alpha	104.46±5.24	47.74±6.97*	2.19	0.0005
192178	L12447	Mus musculus insulin-like growth factor binding protein 5	288.97±8.47	141.67±5.61	2.04	0.0005
192306	X77953	R.norvegicus ribosomal protein S15a.	415.77±54.08	204.19±25.03	2.04	0.005
192129	M38188	Human unknown protein from clone pHGR74	114.72±10.98	57.47±11.64*	2.00	0.0025
192339		Novel	83.94±6.26	42.42±7.75*	1.98	0.001
191857	L00111	Rat CGRP	900.1±45.83	459.99±35.39	1.96	0.0005
192203	AF059486	Mus musculus putative actin-binding protein DOC6	861.16±32.58	448.32±68.77	1.92	0.0005
192351	U25844	Mus musculus serine proteinase inhibitor (SPI3)	271.95±30.44	142.81±6.93	1.90	0.0025
191837	M29472	Rattus norvegicus mevalonate kinase	94.44±9.63	51.83±5.95*	1.82	0.0025
191628		Novel	635.92±73.01	363.86±11.53	1.75	0.005
192175		Novel	181.28±13.23	105.36±10.39	1.72	0.0005
192284		Novel	188.28±13	110.53±7.27	1.70	0.0005
192330	Y10386	MMC1INH M.musculus C1 inhibitor	134.88±11.01	79.3±5.51	1.70	0.0005
192199	D42137	Rat annexin V gene	439.57±13.62	265.21±14.97	1.66	0.0005
192011	M98194	Rat extracellular signal-regulated kinase 1	319.35±32.79	194.88±6.83	1.64	0.005
192206	U59673	Rattus norvegicus 5HT3 receptor	139.96±4.07	85.48±6.17	1.64	0.0005
192167	U23146	Rattus norvegicus mitogenic regulation SSeCKS	456.44±13.34	300.71±23.25	1.52	0.0005
191848	M93056	Human mononcyte/neutrophil elastase inhibitor	125.16±14.76	82.56±15.38	1.52	0.05
192309		Novel	463.17±45.37	308.05±25.45	1.50	0.01

Table 3

PRI ID	GB	Description	Mean±S.E.M.(Small)	Mean±S.E.M. (Large)	Ratio	p
192393	M25638	Rat smallest neurofilament prot in (NF-L)	63.3±6.12	551.56±34.94	8.71	0.0005

191624	M14656	Rat osteopontin	53.4±4.11*	218.52±22.81	4.09	0.0005
192157	J04517	Rat high molecular weight neurofilament (NF-H)	475.86±18.59	1319.77±50.3	2.77	0.0005
192282	Z12152	R.norvegicus neurofilament protein middle	75.93±3.75	206.55±9.92	2.72	0.0005
192378	D87445	Human KIAA0256	30.26±2.66*	77.42±17.52	2.56	0.025
192283		Novel	50.9±3.45*	128.56±6.86	2.53	0.0005
192125	V00681	R.norvegicus mitochondrial genes for 16S rRNA, tRNA	186.5±14.61	445.82±23.95	2.39	0.0005
191851	X51396	Mouse MAP1B microtubule-associated protein	90.84±5.91	215.55±21.35	2.37	0.0025
192424	M91808	Rattus norvegicus sodium channel beta-1	83.99±7.93	194.88±20.61	2.32	0.0025
191862	S67755	hsp 27=heat shock protein 27 [rats, Sprague-Dawley)	144.74±10.14	265.94±19.44	1.84	0.0005
192016	L10426	Mus musculus ets-related protein 81 (ER81)	43.85±1.89*	80.04±7.16	1.83	0.0025
192228		Novel	28.9±1.11*	52±3.41	1.80	0.0005
192411	M21551	Human neuromedin B	57.62±5.56*	97.18±6.61	1.69	0.0005
192422		Novel	110.06±11.78	168.52±12.14	1.53	0.0025

EXAMPLE 1

Laser Capture Microdissection (LCM). Two adult female Sprague Dawley rats were used in this study. Animals were anesthetized with Metofane (Methoxyflurane, Cat# 556850, Mallinckrodt Veterinary Inc. Mundelein, IL, USA) and sacrificed by decapitation. Using RNase-free conditions, cervical dorsal root ganglia (DRGs) were quickly dissected out, placed in cryomolds, covered with frozen-tissue embedding medium OCT (Tissue-Tek), and frozen in dry ice-cold 2-methylbutane (~ -60 °C). The DRGs were then sectioned at 7-10 μm in a cryostat, mounted on plain (non-coated) clean microscope slides and immediately frozen on a block of dry ice. The sections were stored at -70 °C until further use.

A quick Nissl (cresyl violet acetate) staining was employed in order to identify the DRG neurons. This was completed as follows. Slides containing sections were quickly loaded on a slide holder, immediately fixed in 100% ethanol for 1 minute followed by rehydration

via subsequent steps of 95%, 70%, 50% ethanol diluted in RNase free deionized H_2O (5 seconds each). Next, the slides were stained with 0.5% Nissl/0.1 M sodium acetate buffer for 1 minute, dehydrated in graded ethanols (5 seconds each) and cleared in xylene (1 min.). Once air-dried, the slides were ready for LCM.

The PixCell II LCM™ System from Acturus Engineering Inc. (Mountain View, CA) was used for laser-capture. Following manufacture's protocols, 2 sets of large and 3 sets small DRG neurons (1000 cells per set) were laser-captured. The criteria for large and small DRG neurons are as follows: a DRG neuron was classified as small if it had a diameter <25 µm plus an identifiable nucleus whereas a DRG neuron with a diameter >40 µm plus an identifiable nucleus was classified as large.

EXAMPLE 2

RNA Extraction of LCM samples. Total RNA was extracted from the LCM samples with Micro RNA Isolation Kit (Stratagene, San Diego, CA) with some modifications. Briefly, after incubating the LCM sample with 200 µl of denaturing buffer and 1.6 µl ß-ME at room temperature for 5 min., the LCM sample was extracted with 20 µl of 2M sodium acetate, 220 µl phenol and 40 µl chloroform: isoamyl alcohol. The aqueous layer was collected and then mixed with 1 µl of 10 mg/ml carrier glycogen, and precipitated with 200 µl of isopropanol. Following 70% ethanol wash and air-dry, the pellet was resuspend in 16 µl of RNase free H2O, 2 µl 10X DNase I reaction buffer, 1 µl RNasin and 1 µl of DNase I, incubated at 37°C for 30 minutes to remove any genomic DNA contamination. Next, the phenol chloroform extraction was repeated as above. The pellet was resuspend in 11 µl of RNase free H2O, 1 µl of which was saved and used as a negative control for reverse transcription PCR (no RT control), and the remaining (10 µl) was processed for RT-PCR and RNA amplification.

EXAMPLE 3

Reverse Transcription(RT) of RNA. First stand synthesis was completed by adding together 10 μl of purified RNA from above and 1 μl of 0.5 mg/ml T7-oligodT primer (5'TCTAGTCGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGT_{21}-3'). Primer and RNA were incubated at 70°C 10 minutes, followed by 42°C for 5 minutes. Next, 4 μl of 5X first strand reaction buffer, 2 μl 0.1M DTT, 1 μl 10mM dNTPs, 1 μl RNasin and 1 μl Superscript II (Gibco BRL) were added and incubated at 42°C for one hour. Next, 30 μl second strand synthesis buffer, 3 μl 10 mM dNTPs, 4 μl DNA Polymerase I, 1 μl *E. coli* RNase H, 1 μl *E. coli* DNA Ligase and 92 μl of RNase free H_2O were added and incubated at 16°C for 2 hours, followed by 2 μl of T4 DNA Polymerase at 16°C for 10 minutes. Next, the cDNA was phenol-chloroform-extracted and washed 3X with 500 μl of H_20 in a Microcon-100 column (Millipore). After collection from the column, the cDNA was dried down to 8 μl for in-vitro transcription.

EXAMPLE 4

T7 RNA Polymerase Amplification (aRNA). *Ampliscribe* T7 Transcription Kit (Epicentre Technologies) was used: 8 μl double-stranded cDNA, 2 μl of 10X *Ampliscribe* T7 buffer, 1.5 μl of each 100 mM ATP, CTP, GTP and UTP, 2 μl 0.1 M DTT and 2 μl of T7 RNA Polymerase, at 42°C for 3 hours. The aRNA was washed 3X in a Microcon-100 column, collected, and dried down to 10 μl.

Subsequent Rounds of aRNA Amplification. 10 μl of aRNA from first round amplification was mixed together with 1 μl of 1mg/ml random hexamers (Pharmacia), 70°C for 10 minutes, chilled on ice, equilibrated at room temperature for 10 minutes, then 4 μl 5X first stand buffer, 2 μl 0.1M DTT, 1 μl 10mM dNTPs, 1 μl RNasin and 1 μl Superscript RT II were added and incubated at room temp. for 5 minutes followed by 37°C for 1 hour. Then, 1 μl of RNase H was added and incubated at 37°C for 20 min. For second strand cDNA synthesis, 1 μl of 0.5 mg/ml T7-oligodT primer was added and

incubated at 70°C for 5 minutes, 42°C for 10 minutes. Next, 30 μl of second strand synthesis buffer, 3 μl 10mM dNTPs, 4 μl Polymerse I, 1 μl *E. coli* RNase H, 1 μl *E. coli* DNA Ligase and 90 μl of RNase free H2O were added and incubated at 37°C for 2 hours. Then 2 μl of T4 DNA Polymerase was added at16°C for 10minutes. The double strand of cDNA was extracted with 150 μl of phenol chloroform to get ride of protein and purified with Microcon-100 column (Millipore) to separate from the unincorporated nucleotides and salts. The cDNA is ready for second round T7 in vitro transcription as above and then a subsequent third round aRNA amplification.

EXAMPLE 5

Microarray Design. The cDNAs present on the chip were obtained from two separate differential display[21] experiments. First, we preformed a screen to clone mRNAs preferentially expressed in DRG versus brain, kidney and liver. Second, a screen was done to identify/clone mRNAs with decreased or increased concentration in ipslateral ("treated") lumbar 5 and 6 DRGs that were tightly ligated distal to the dorsal root ganglion ("Chung" model[22]) versus contralateral ("control") lumbar 5 and 6 DRGs.

Microarray Printing. 477 clones in vector PCR 2.1 from our previous differential display studies (as described above) were printed on silylated slides (CEL Associates). cDNAs were PCR-amplified with 5' amino-linked primers and purified with Qiagen 96 PCR Purification Kits. The print spots were about 125 μm in diameter and were spaced 300 μm apart from center to center. 30 plant genes were also printed on the slides as a control for non-specific hybridization (gift from Mark Schena)

EXAMPLE 6

Microarray Probe Synthesis. Cy3 labeled cDNA probes were synthesized from aRNA of LCM DRGs with Superscript Choice System for cDNA Synthesis (Gibco BRL). In brief, 5 μg aRNA, 3 μg random hexamer were mixed in a total volume of 26 μl (containing RNase free H_2O), heated to 70°C for 10 minutes and chilled on ice. Then,10

μl first strand buffer, 5 μl 0.1MDTT, 1.5 μl RNasin. 1 μl 25mMd(GAT)TP, 2 μl 1mM dCTP, 2 μl Cy3-dCTP (Amersham) and 2.5 μl Superscript RT II were added and incubated at room temp. for 10 minutes and then 37°C for 2 hours. To degrade the aRNA template, 6 μl 3N NaOH was added and incubated at 65°C for 30 minutes. Then, 20 μl 1M Tris-HCl pH 7.4, 12 μl 1N HCl and 12 μl H_2O were added. The probes were purified with Microcon 30 Columns (Millipore) and then with Qiagen Nucleotide Removal Columns. The probes were vacuum dried and resuspend in 20 μl of hybridization buffer (5X SSC, 0.2% SDS) containing mouse Cot1 DNA (Gibco BRL).

Microarray Hybridization and Washes. Printed glass slides were treated with sodium borohydrate solution (0.066M NaBH4, 0.06M Na AC) to ensure amino-linkage of cDNAs to the slides. Then, the slides were boiled in water for 2 minutes to denature the cDNA. Cy3 labeled probes were heated to 99°C for 5minutes, room temperature for 5 minutes and applied to the slides. The slides were covered with glass cover slips, sealed with DPX (Fluka) and hybridized at 60°C for 4-6 hours. At the end of hybridization slides were cooled to room temperature. The slides were washed in 1X SSC, 0.2% SDS at 55°C for 5 minutes, 0.1X SSC, 0.2% SDS at 55°C for 5 minutes. After a quick rinse in 0.1X SSC, 0.2% SDS, the slides were air-blown dried and ready for scanning.

Microarray Quantitation. cDNA microarrays (i.e., microscope slides) were scanned for cy3 fluorescence using the ScanArray 3000 (General Scanning, Inc.). ImaGene Software (Biodiscovery, Inc.) was then subsequently used for quantitation. In total, 15 chips were processed, with 3 chips/neuronal set (see text). Briefly, the intensity of each spot (i.e., cDNA) was corrected by subtracting the immediate surrounding background. Next, the corrected intensities were normalized for each cDNA (i.e., spot) with the following formula: intensity (background corrected) / 75-percentile value of the intensity of the entire chip x 1000. To determine "non-specific" nucleic acid hybridization, 75-percentile values were calculated from the individual averages of each plant cDNA (for a total of 30 different cDNAs) from each neuronal set. The overall 75-percentile value for S1, S2 and S3 = 48.68 and for L1 and L2 = 40.94.

Statistical Analyses. To assess correlation of intensity value for each cDNA between individual sets of neurons (e.g., S1 vs. S2 in Fig. 2B) or between two neuronal subtypes (i.e., S1, S2 and S3 vs. L1 and L2 in Fig. 2B), scatter plots were used and linear relationships were measured. The coefficient of determination, R^2, that was calculated, indicates the variability of intensity values in one group vs. the other.

To statistically determine whether or not intensity values measured from microarray quantitation are true signals, each intensity is compared, via a one-sample t-test, to the 75-percentile value of 30 plant cDNAs that are present on each chip (representing non-specific nucleic acid hybridization). Values not significantly different from the 75-percentile value that are in presented in Table 1 and 2 and so denoted. To determine which cDNAs are statistically significant in their differential gene expression between large and small neurons, the intensity for each cDNA from neuronal sets for large neurons (L1 and L2) and small neurons (S1,S2, and S3) were grouped together respectively and intensity values were averaged for each corresponding cDNA. Two-sample *t* test for one-tailed hypotheses was used to detect a gene expression difference between small neuron and large neurons.

EXAMPLE 7

***In Situ* Hybridization.** *In situ* hybridization was carried out as previously described.[23] Briefly, cDNAs were subcloned into pBluescript II SK (Stratagene) linearized and ^{35}S-UTP was incorporated via in-vitro transcription with T7 or T3 RNA polymerase. The probes were then purified with Quick Spin™ Columns(Boehringer Mannheim). Probes (10^7 cpm's /probe) were hybridized to 10 µm, 4% paraformaldehyde-fixed rat DRG sections which were mounted on Superfrost Plus slides (VWR). After overnight hybridization at 58°C and post-washes, the slides were exposed to film for primary data. Subsequently the slides were coated with Kodak liquid emulsion NTB2 and exposed in light-proof boxes for 1-2 weeks at 4°C. The slides were developed in Kodak Developer D-19, fixed in Kodak Fixer and Nissl stained for expression analysis.

Under light field microscopy, mRNA expression levels of specific cDNAs were semi-quantitatively analyzed. This was done as follows: no expression (-, grains were <5 fold of the background); weak expression (±, grains were 5-10 fold of the background); low expression (+, grains were 10-20 fold of the background); moderated expression (++, grains were 20-30 fold of the background); strong expression (+++, grains were >30 fold of the background). The percentage of small or large neurons expressing a specific mRNA was obtained by counting the number of labeled (above background) and unlabeled cells from either large or small neurons from four sections (at least 200 cells were counted).

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WHAT IS CLAIMED IS:

1. A method, comprising:

a) selecting and attaching cells to a substrate by laser capture microdissection, and isolating the captured cells from the remaining cells;

b) extracting the RNA from the isolated captured cells and amplifying the RNA;

c) producing cDNA from the amplified RNA of step b) and labelling the cDNA with a detectable label to produce labeled cDNA;

d) hybridizing the labeled cDNA of step c) with DNA probes on an immobilized DNA microarray; and

e) determining which immobilized DNA on the microarray hybridized with labeled cDNA, quantitatively and/or qualitatively.

2. A method, comprising:

a) selecting and attaching cells to a substrate by laser capture microdissection, and isolating the captured cells from the remaining cells;

b) extracting the RNA from the isolated captured cells and amplifying the RNA and labelling the amplified RNA with a detectable label to produce labeled amplified RNA;

c) hybridizing the labeled amplified RNA of step b) with immobilized DNA on an immobilized DNA microarray; and

d) determining which immobilized DNA on the microarray hybridized with labeled amplified RNA, quantitatively and/or qualitatively.

3. A method, comprising:

a) selecting and attaching cells to a substrate by laser capture microdissection, and isolating the captured cells from the remaining cells;

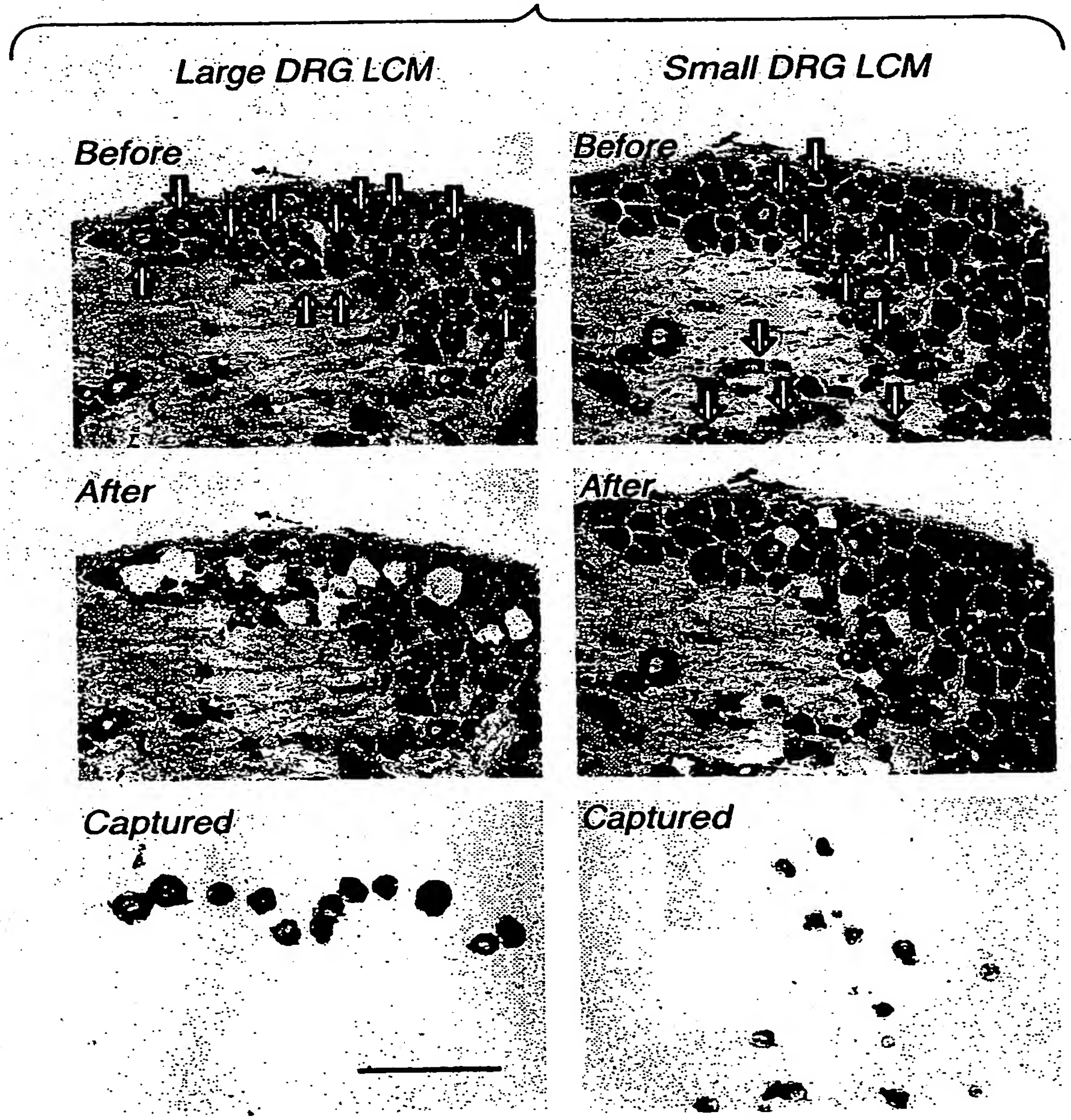
b) extracting the RNA from the isolated captured cells and amplifying the RNA and labelling the amplified RNA with a detectable label to produce labeled amplified RNA;

c) producing cDNA from the amplified RNA of step b) and labelling the cDNA with a detectable label to produce labeled cDNA;

d) hybridizing the labeled amplified RNA and/or labeled cDNA with DNA on an immobilized DNA microarray; and

e) determining which immobilized DNA on the microarray hybridized with labeled cDNA, quantitatively and/or qualitatively.

FIG. 1


Large DRG LCM
Small DRG LCM
Before
Before
After
After
Captured
Captured

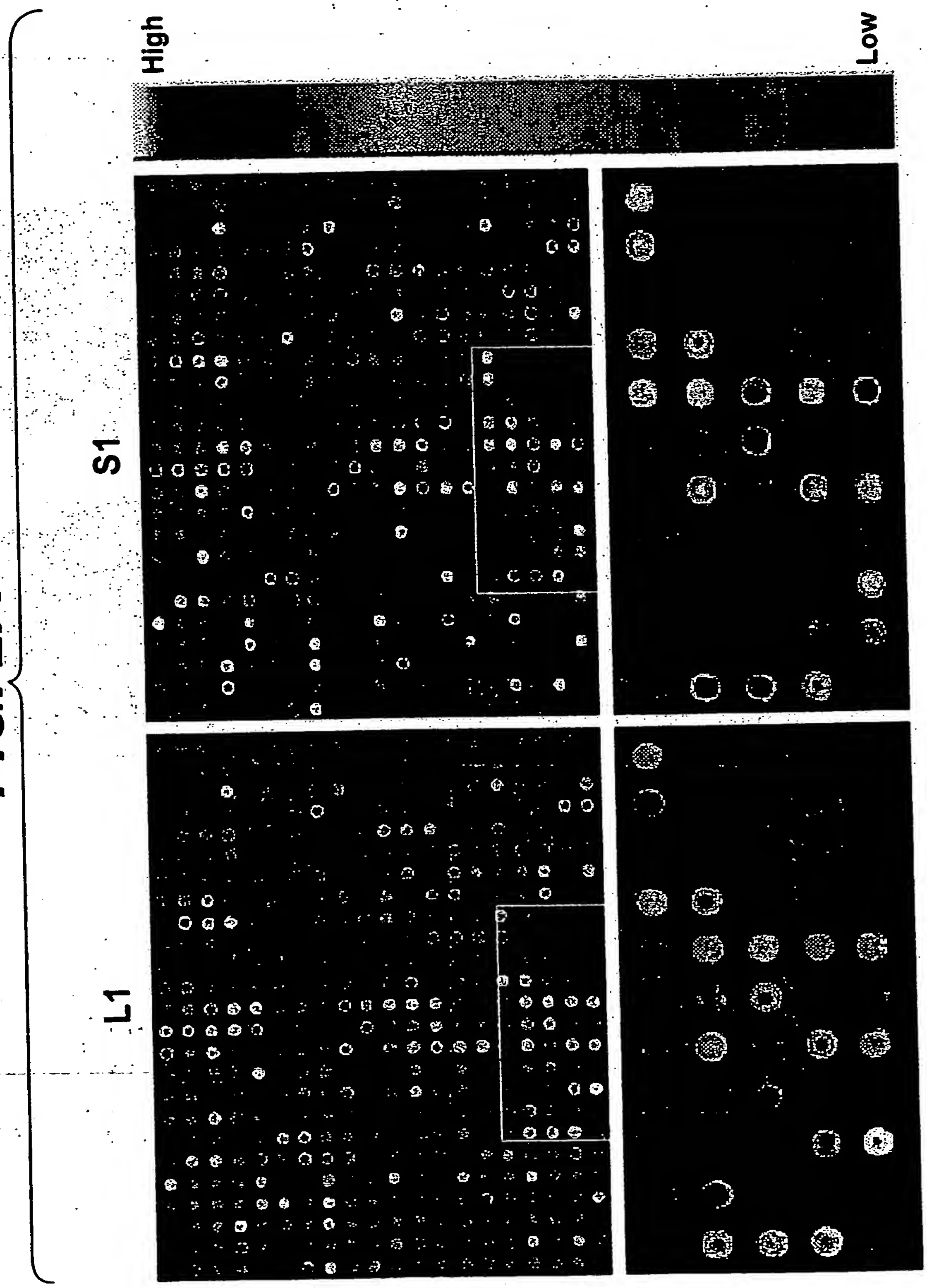


FIG. 2A

FIG. 2B

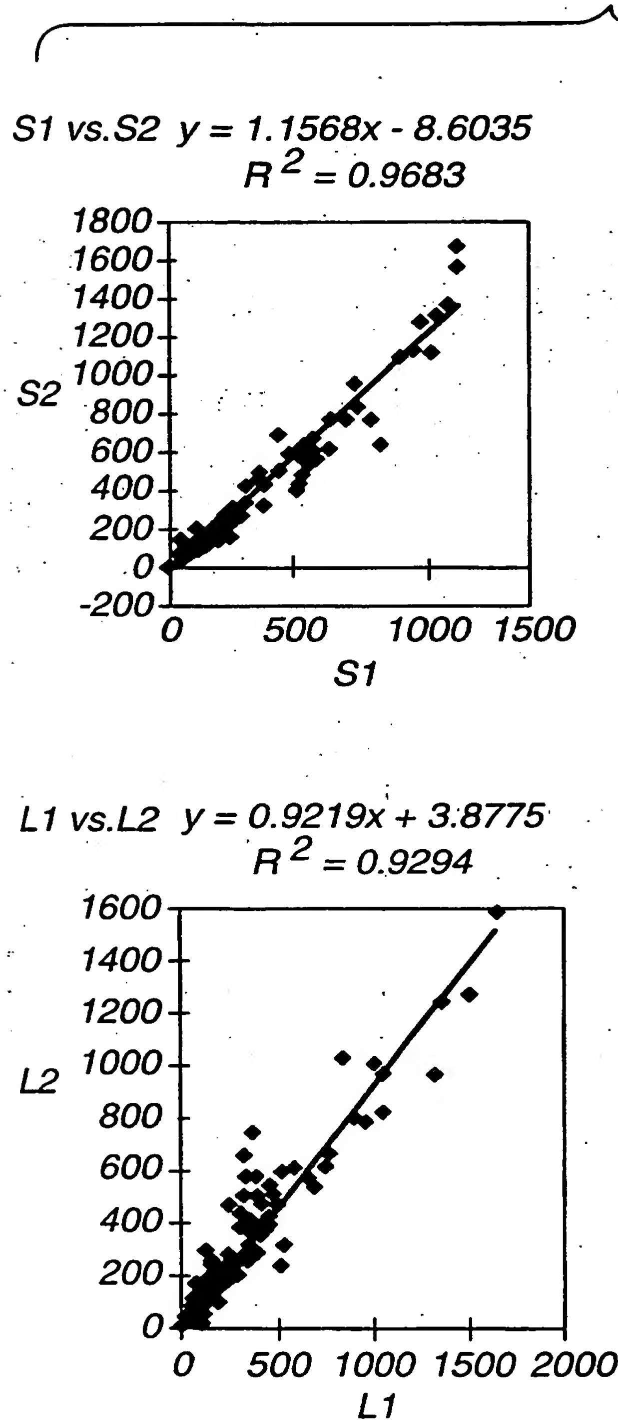
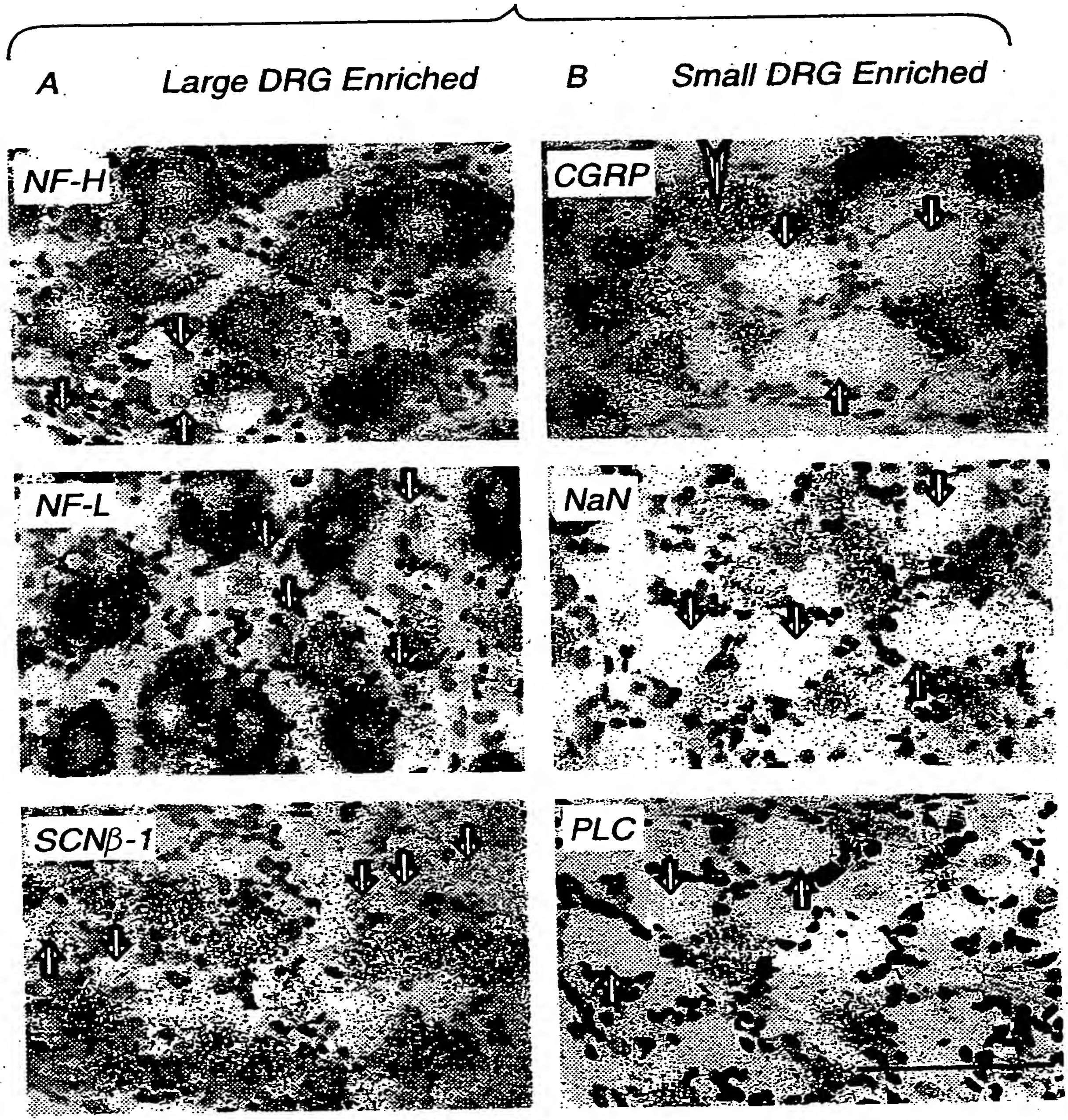

S1 vs.S2 y = 1.1568x - 8.6035
R 2 = 0.9683
S2
S1
1800
1600
1400
1200
1000
800
600
400
200
0
-200
0 500 1000 1500
S1 vs.S3 y = 0.9617x + 1.0612
R 2 = 0.9399
S3
S1
1600
1400
1200
1000
800
600
400
200
0
0 500 1000 1500
L1 vs.L2 y = 0.9219x + 3.8775
R 2 = 0.9294
L2
L1
1600
1400
1200
1000
800
600
400
200
0
0 500 1000 1500 2000
S vs.L y = 0.7584x + 19.397
R 2 = 0.6789
L
S
1800
1600
1400
1200
1000
800
600
400
200
0
0 500 1000 1500

FIG. 3


A
Large DRG Enriched
B
Small DRG Enriched
NF-H
CGRP
NF-L
NaN
SCNβ-1
PLC

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/09594

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68; C12P 19/34; G01N 1/00; C07H 21/04
US CL :435/6, 91.1,91.2, 91.21; 436/174; 536/24.3, 24.31, 24.33;
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1,91.2, 91.21; 436/174; 536/24.3, 24.31, 24.33;

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98/35216 A1 (ARCTURUS ENGINEERING, INC.) 13 August 1998 (13.08.98), see whole document especially abstract.	1-3
A,P	US 5,859,699 A (BAER et al) 12 January 1999, see entire document especially columns 1-2.	1-3
Y	SIMONE, N.L. et al. Laser-capture microdissection: opening the microscopic frontier to molecular analysis. TIG. July 1998, Vol. 14, No. 7, pages 272-276, entire document.	1-3
Y	EMMERT-BUCK, M.R. et al Laser Capture Microdissection. Science. 08 November 1996, Vol. 274, pages 998-1001, entire document.	1-3

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 JUNE 1999	01 NOV 1999

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Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	JEFFREY SIEW JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ZHAO, N. et al. High-density cDNA filter analysis: a novel approach for large-scale, quantitative analysis of gene expression. Gene. 1995, Vol. 156, pages 207-213, entire document.	1-3
Y	WO 89/10977 A1 (ISIS INNOVATION LIMITED) 16 November 1989 (16.11.89), see whole document	1-3
Y	SCHENA, M. et al. Quantitative Monitoring of Gene Expression Patterns with a Complementary DNA Microarray. Science. October 1995, Vol. 270, pages 467-470, entire document.	1-3
A , P	LUO, L. et al. Gene Expression profiles of laser-captured adjacent neuronal subtypes. Nature Medicine. January 1999, Vol. 5, No.1, pages 117-122.	1-3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/09594

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS,EPO, BIOSIS, MEDLINE,CANCERLIT, BIOTECHDS,LIFESCI, CAPLUS,EMBASE
search terms: laser capture microdissect, LCM, capture cells, differential gene expression, label, amplified rna,cdna, array, chip, probe, dorsal root ganglia, neurons,